DEVELOPMENT OF *Cochliomyia macellaria* ON EQUINE AND PORCINE STRIATED MUSCLE TISSUE AND ADULT ATTRACTION TO LARVAL RESOURCE

AThesis

by

STACY ANN BOATRIGHT

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

ENTOMOLOGY

August 2009

Major Subject: Entomology

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Approved by:

Chair of Committee,	Jeffery K. Tomberlin
Committee Members,	Tracy Cyr
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Head of Department,	Kevin Heinz

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ABSTRACT

Development of *Cochliomyia macellaria* on Equine and Porcine Striated Muscle Tissue and Adult Attraction to Larval Resource. (August 2009)

Stacy Ann Boatright, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Jeffery K. Tomberlin

Cochliomyia macellaria is commonly utilized in estimating the period of insect activity (PIA) on a corpse due to its rapid colonization time of fresh remains, active oviposition during daylight, and its abundance throughout the southern United States. The primary objective of this study involved *C. macellaria* reared on either equine or porcine striated muscle tissues at 21°C, 24°C, and 27°C. *C. macellaria* required approximately 35% more time to complete development when reared at 21°C instead of 27°C. Larval growth and weight gain over time did not differ between larvae reared on equine versus porcine muscle tissue. This study is the second in the United States to examine the development of *C. macellaria* and is the first to examine development of this species on muscle tissue from different vertebrate species. These data could provide significant information for myiasis and neglect cases of humans and animals. The second study addressed whether or not adult male and female *C. macellaria* blow flies demonstrated a preference for their larval resource based on olfactory responses in a Y-tube olfactometer. Flies were reared either on beef liver or testicles. Initial and final responses as well as residence time in each arm of the olfactometer over a five minute

period were recorded. An additional experiment for flies reared on liver assessed adult male and female preference for resources with and without conspecific larvae present. Seven to ten day old blow flies were used in the olfactometer experiments. Only final choice of females reared on liver demonstrated a significant ($\alpha = 0.05$) attraction for larval resource. All flies reared on beef testicles showed a preference for their larval resource in initial and final choices and residence time. No significant difference was determined for the parameters measured for either sex in response to the presence of conspecific larvae. Data indicate that adult male and female *C. macellaria* flies are generalists and do not show a preference for their larval resource or for resources inoculated with conspecific larvae.

DEDICATION

I wish to dedicate this thesis to my amazing grandparents, Sophie and Bill Pribble, better known as Nana and Poppy. I grew up in your home, and I have always considered you to be my second set of parents. You instilled important morals and life lessons in me that will forever be ingrained into my soul. Without you both in my life, I would not be the ambitious woman that I am today. From my first part in a play as an angel in preschool at St. Matthews to my college graduation from Texas A&M University, you have always been there to support me in everything that I pursue. I love you both and am so grateful for all of your everlasting support

Nana, you taught me the importance of treating all others as I would want to be treated. You showed me that presenting myself in a positive and optimistic way will undoubtedly earn the respect of others. Your endless encouragement for me to keep striving to reach my goals has resulted in great accomplishments. Thank you for always being there for me in everything that I do. Words cannot express how fortunate and blessed I am to have had you as such an integral part of my life for all of these years.

Poppy, you taught me the importance of honesty and fairness in all aspects of life, no matter the circumstances. You also showed me that ambition and an outstanding work ethic will lead to the greatest of accomplishments. I missed you at my first college graduation, and I will miss you even more as I graduate with my Master's degree, but I know you have been watching me from heaven throughout my entire journey.

ACKNOWLEDGEMENTS

I would like to extend my deepest and sincerest thanks to my committee chairman, Dr. Jeffery Tomberlin, for his unyielding guidance and support throughout this process. I thank him for providing me the wonderful opportunity to be a teaching assistant every semester during graduate school in order to receive funding. It has been an honor and privilege to work in his laboratory for the past two years, and I can never thank him enough for providing me with so many opportunities to give lectures and present my research at national conferences. I honestly could not have asked for a more actively-involved and supportive advisor for graduate school, and I thank Jeff for encouraging and believing in me throughout this entire process. I am certain that he and I will always keep in touch and remain friends for many years, regardless of where my career may take me.

I would also like to thank my committee member Dr. Tracy Cyr for her excellent advice, ideas, and support; it was an honor and a privilege working with her. I also extend my thanks to my committee member Dr. Bradleigh Vinson for his helpful ideas and support throughout this process, as his vast knowledge of insect behavior aided me in fully understanding the concepts presented in my secondary objective.

I also appreciate the help from everyone in the F.L.I.E.S. laboratory, especially that which came from Micah Flores. I could not have wished for a more hard-working and kind lab partner. His willingness to go out of his way to help me throughout the past two years will forever be appreciated. I am also grateful for all of Micah's support and

advice, and I know that my graduate school experience would not have been the same without him. I want to also thank Adrienne Brundage for allowing me to be a teaching assistant for her Forensic Entomology class during my last semester.

I am also gracious to Jordan Coburn, as his knowledge of statistics and the SPSS program that I used when analyzing my data was incredible. All of the hours that he took out of his own busy schedule to sit down and help me are much appreciated. I honestly do not know how I could have completed all of my analyses without Jordan's SPSS tutoring sessions. He saved me an incredible amount of time and made the over all analysis process far less stressful than it would have been otherwise.

I also wish to thank Dr. Everett Simmons at Burnet Road Animal Hospital for inspiring me to pursue a higher education in the field of Entomology and for acting as my mentor throughout my time in graduate school. I appreciate his willingness to help me obtain canine tissue used in my experiments, and I am grateful for all of his support and encouragement. I would also like to extend my thanks to Dr. Gayne Fearneyhough at the Texas Veterinary Medical Diagnostic Laboratory for donating and screening the equine tissue utilized in my thesis experiments.

One of the major highlights of my time in graduate school was teaching labs for Veterinary Entomology; therefore, I wish to thank Dr. Darrell Bay for accepting me as his teaching assistant for all four semesters. This was a great experience because it not only provided my funding for school, but it taught me a great deal about Veterinary Entomology in the process of teaching.

Most importantly, I would like to acknowledge my amazing family, Chris and Marilyn Kennison and Sophie Pribble, for all of their incredible support and encouragement. My mother inspired me to obtain a higher education and never ceased to believe that I could achieve my goals. I cannot express how much I appreciate her and Chris's financial support that allowed me the opportunity to earn my Master's degree. Chris has always treated me as his own daughter and financially supported me throughout my college career, which mean the world to me. I would not have been able to complete graduate school without all of the love and endless support from Nana, Chris, and my mother. I am truly blessed and fortunate to have such an amazing family who has always encouraged me to pursue my dreams and be the successful woman that I will surely become after I graduate.

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CHAPTER I

INTRODUCTION

Forensic entomology is the utilization of insects and other arthropods as evidence in both civil and criminal legal investigations (Williams and Villet 2006). The broad scope of this field can be broken down into its three subdivisions which are urban, stored-product, and medicolegal entomology (Archer 2007). Urban entomology, as it relates to forensics, encompasses arthropods colonizing human structures (Catts and Goff 1992). Stored product entomology involves the contamination of food products by arthropods (Catts and Goff 1992). The third subdivision of forensic entomology, and the focus of this study, is medicolegal.

History of Medicolegal Entomology

Forensic, or medicolegal, entomology has become increasingly popular over recent years. The first case involving the use of insects as evidence in solving a crime occurred in 1235 A.D. This investigation was described by Sung Tz'u, a Chinese lawyer and death investigator, who published a text entitled *The Washing Away of Wrongs* (Catts and Goff 1992). In this book, he discussed a murder on a rice farm. It was believed through observation that the victim's multiple slash wounds were inflicted with

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This thesis follows the style of the Journal of Medical Entomology.

a sickle, so Sung Tz'u ordered all farmers in the area to assemble and present their sickles. Although the culprit had washed the blood from his sickle, flies accumulated on it due to blood particles still being present. The murderer saw this accumulation of flies as a bad omen and confessed his crime.

During the mid 19th century in Europe, forensic entomology was utilized by Bergeret d'Arbois in a death investigation of a child's mummified remains discovered behind the mantelpiece in a home located in Paris, France (Catts and Goff 1992). Bergeret applied the same concepts of analyzing arthropod succession on human remains that are used presently. Megnin also examined arthropod evidence from humans by studying decomposition as it related to insect activity, concluding that different insect species colonize a corpse in eight "waves" (Keh 1985). Megnin's work involved the postmortem interval (PMI) concept, although he did not assess the ages of various insect species when making his determinations.

Jerry Payne (1965), at Clemson University, observed the physical changes that occur during pig carcass decomposition in relation to arthropods, also referred to as faunal succession (Benecke 2004). As a result of these studies, Payne (1965) developed six stages of decomposition as they apply to insect colonization, which are still utilized today.

Applications of Medicolegal Entomology

One aspect of medicolegal entomology involves necrophagous arthropods colonizing and feeding upon living and deceased human and animal tissue. Insects found

on human remains may be utilized to estimate a period of insect activity (PIA). Locating the most mature insect specimen at the scene of corpse discovery may reveal the time elapsed between initial insect colonization and the finding of the decedent. Because insects are normally the first to arrive at a crime scene, showing up within minutes of death, this PIA may also coincide with an accurate PMI estimate (Archer 2007). However, the term "PMI" must be used with caution, as insect colonization may be delayed if conditions restrict access to the body (Benecke 2004). Insects may also be used to determine whether a body has been moved from its location of initial colonization (Archer 2007). A third application of medicolegal entomology involves the extraction of human DNA from the gut material of the insects collected from the corpse (Benecke 2004). Identification of such non-insect DNA is useful in cases where maggots, but no corpse, are found at a crime scene, and the insects' last meal is identified (Benecke 2004). Insect colonization of human remains follows a predictable sequence which is initiated in the natural orifices of the body. Decomposition progresses from the head and pelvic area towards the abdominal region and out the extremities. Any region of the corpse where putrefaction is disproportionately advanced in comparison to the rest of the body (i.e. chest area having heavy larval colonization in comparison to the head) would be indicative of trauma to that region before or shortly after death and should be examined in detail, as it might be indicative of antemortem wounds (Campobasso and Introna 2001). Insects feeding on tissue that contains foreign substances, such as illegal or prescription drugs, or poison, will often store these substances in their own bodies (Benecke 2004). Therefore, entomotoxicology is another

medicolegal application in which the gut contents of the arthropods sampled from human remains can be analyzed for foreign substances. Time of year when the decedent was colonized can be determined based on the arthropod species or artifacts collected from the remains due to many species being active only during certain periods of the year (Archer 2007).

Colonization of living humans and animals may be indicative of neglect, abuse, and/or sexual molestation (Anderson and Huitson 2004). Myiasis, the infestation and subsequent feeding upon living tissue, organs, or bodily fluids of a vertebrate host by dipteran larvae, can be used as evidence in cases of suspected neglect or abuse (Anderson and Huitson 2004). Myiasis most commonly occurs in elderly or very young individuals, or in pets and livestock when there is an open wound or fecal matter on the animal for an extended time period (Anderson and Huitson 2004).

Variation in Development Data

Forensic entomologists depend on development data from laboratory studies to estimate PIA for blow flies (Diptera: Calliphoridae) on human remains (Tarone and Foran 2006). Although pre-existing data sets within a blow fly species demonstrate similar development times, all do vary (Tarone and Foran 2006). Such variation could be due to a number of factors, such as laboratory protocols and genetic variation of a given fly species between geographic locations. For example, Tarone and Foran (2006) found that environmental conditions greatly influenced the development of the blow fly *Lucilia sericata* (Meigen) (Diptera: Calliphoridae). Their findings demonstrate the need for

multiple development data sets for each species of blow fly, across geographic regions, as well as for a standardization of laboratory rearing techniques.

Development data utilized by forensic entomologists in estimating PIA are obtained from studies of growth of larvae that are usually reared on beef liver (Clark et al. 2006). However, it has been demonstrated that species of origin on which the larvae feed may significantly alter the larval growth rate (Clark et al. 2006). For example, Clark et al. (2006) compared the development of *L. sericata* on lung, liver and heart, from both bovine and porcine species. Their study determined larvae grew significantly larger on porcine liver compared to bovine liver, with an overall difference in the detransformed mean lengths being 0.63 mm. Larvae in this study grew faster and larger when reared on lung and heart compared to liver. Larvae reared on porcine lung tissue compared to porcine liver resulted in the larvae feeding on lung entering the prepual stage 31 hours earlier and growing approximately 2mm longer (Clark et al. 2006). For forensic entomologists, such results emphasize the need for development data sets across tissue types, as well as across various species; they also demonstrate the importance of recording the specific tissue from which blow fly larvae are removed from a corpse at a crime scene (Clark et al. 2006).

In addition to development data sets, forensic entomologists also depend on succession studies to predict the PIA of decomposing remains (Payne 1965). Data produced by such studies aid in estimating the PIA for corpses in later stages of decomposition, such as the skeletal stage (Wang et al. 2008). However, predicting initial colonization from succession studies is possible at this date. Some researchers

hypothesize that various environmental factors, such as wind, rain, and cold can delay colonization (Campobasso et al. 2001); however, circumstances do occur where vertebrate carrion is not colonized for hours or days under "ideal" conditions. By understanding the factors that attract adult flies to initially locate and subsequently colonize carrion, forensic entomologists will gain a better understanding of blow fly biology and, therefore, be able to develop more accurate PIA estimations.

The precise mechanisms that govern attraction to a resource and site selection by blow flies are not fully known, but they are presumed to be chemical and mechanical in nature (Archer and Elgar 2003a). The presence of water, egg clutches of other females, and certain water-soluble proteins have been discovered to attract adult blow flies and stimulate oviposition in the females (Barton-Browne 1962). Little research has assessed the effect of conspecific larvae on a resource as it relates to adult blow fly attraction, although Eisemann and Rice (1987) did conclude that microbial products associated with feeding maggots have an attractive effect on gravid female *L. cuprina*.

To date, no one has examined the relationship between adult blow flies and their attraction to their larval resource. Will flies reared on one resource demonstrate a higher level of attraction to that resource than an alternative that might be present? I hypothesize that *C. macellaria* adults show a greater attraction to resources similar to what they experienced during the larval stage. Furthermore, I hypothesize that attraction also is regulated by the presence or absence of conspecifics. Forensic implications exist in relation to understanding the mechanisms by which blow flies are attracted to carrion, as maggots from such flies are most frequently utilized by forensic entomologists as

evidence in estimating minimum PMI in investigations (Archer and Elgar 2003a). Associative learning and the chemical legacy hypotheses represent two explanations for colonization patterns of resources in nature. These hypotheses might offer some explanation why decomposing vertebrate resources present under "ideal" conditions are not colonized immediately by blow flies.

Learning

Learning can be described as any modification in behavior due to experience (McCall and Kelly 2002). The ability to learn is an essential mental function which depends upon an organism's ability to perceive information. The ability to acquire, store, and retrieve information from an animal's experiences is genetically based, but the information is acquired within that animal's life (McCall and Kelly 2002).

Associative Learning for Locating Resources

The capacity to learn is universal among animals (Hendel et al. 2005). Because animals live in a world of dynamic stimulus events, innate behavioral responses to such events are imperative to that animal's survival (Daly and Smith 2000). Under many circumstances, there are advantages for the animal's responses to display some degree of plasticity based on prior experience (Daly and Smith 2000). For insects, such advantages include the ability to optimize foraging, find potential mates, and locate hosts (Daly et al. 2001).

Associative learning has been extensively studied in a wide range of arthropods, including moths (Lepidoptera: Sphingidae), bees (Hymenoptera: Apidae), and mosquitoes (Diptera: Culicidae), (Daly et al. 2001, McCabe et al. 2007, Tomberlin et al. 2006). However, few have undertaken studies that seek to assess the ability of forensically-important arthropods to learn. Because blow flies (Diptera: Calliphoridae) are generally the first to arrive at human and animal remains, understanding their ability to memorize and learn would undoubtedly prove to be beneficial in the field of forensic entomology. Although a variety of associative learning research studies involving blow flies are currently underway, a few publications have paved the way for such studies (McGuire 1981, Nelson 1971, Dethier 1992).

One research experiment assessed the central excitatory state (CES) response in the blow fly, *Phormia regina* (McGuire 1981). The CES is a change in the central nervous system of a fly that occurs after sugar stimulation so that the fly would respond to some inappropriate stimulus by extending its proboscis (McGuire 1981). It has been suggested that prolonged mounting or immobilization of flies may also induce the CES response, and that such a response is involved in the conditioning of blow flies (Nelson 1971). In short, Nelson implied that classical conditioning may be an extension of the CES response, although her evidence was inconclusive.

The "learned response" of proboscis extension may be due to the CES alone under conditions of stressful or prolonged mounting, or the CES may be a predisposition for learning (McGuire 1981). McGuire performed a study that assessed the relationship between classical conditioning and the CES in the blow fly, *Phormia regina*. Response

to selection for CES was very fast in his experiments, suggesting that behavioral differences in CES are correlated with differences at a single locus (McGuire 1981). McGuire commented that CES selection experiments are a first step in the analysis of classical conditioning in blow flies.

Dethier performed experiments that assessed behavioral habituation in *Phormia regina* (Dethier 1992). Behavioral habituation involves an animal's responsiveness to a stimulus to cease (Dethier 1992). His study involved releasing wingless blow flies onto a flat uniform substrate and analyzing their hopping movements, as hopping is an action commonly associated with flight initiation. He noticed that the rate of hopping decreased over time, resembling behavioral habituation. Dethier was able to reestablish hopping of his *P. regina* subjects through tactile stimulation. His study sought to assess the relationship between habituation and physiological variables, such as circadian rhythms or age of the test subject; neither of these variables were shown to attribute to the waning of hopping in the flies (Dethier 1992).

As shown, it has been demonstrated that a variety of insects are capable of learning. However, an extremely limited number of researchers have assessed the concept of learning and memory in forensically-important arthropods, such as members of the family Calliphoridae. By understanding how calliphorid adults effectively locate and exploit certain oviposition sites, such as human corpses, forensic entomologists would be able to better assess the circumstances surrounding the period of insect activity (PIA) in death investigations. Through the implication of associative learning studies of blow flies, forensic entomologists will be able to better interpret why blow flies colonize

certain carrion over others, what volatiles attract the flies to carrion, and what factors affect the choice to oviposit (such as the presence of a predatory species). Understanding what associations are linked to such behaviors will inevitably lead to more accurate estimates of PIA, as well as give researchers insight into the mental capacities of these important insects. However, in recent years, associative learning by adults with larval resources has come under fire.

Chemical Legacy

The assumption that adult insects show a preference for the resource on which they themselves developed as larvae is known as the Hopkins' host-selection principle (HHSP) (Hopkins 1916). HHSP implies that insects experience what is referred to as preimaginal conditioning, meaning that the behavior of an adult insect is conditioned by larval experience (Barron 2001). This theory was widely accepted at the time of its initial proposal in 1916, but has been condemned by researchers in recent years due to the numerous factors that contribute to a behavioral bias for the developmental host insect (Barron 2001). Inducing a food resource or host preferences in adult insects by exposing them as larvae to a specific food source or host has been performed in early demonstrations of preimaginal conditioning (Thorpe 1939, Manning 1967); however, convincing results from such demonstrations in holomotabolous insects are rare (Barron 2001). Because such studies have been received with skepticism for failing to effectively isolate emergent adult insects from their preimaginal environment (Jaenike 1988), the proposal that adults respond to a "chemical legacy" of their larval environment has

become accepted as a more plausible reason for adult insect behavior in response to their larval resource (Corbet 1985).

The chemical legacy theory implies that an adult behavioral change is acquired by insects in the early-emergent stage, rather than preimaginal stage (Barron 2001). This legacy takes into account the persistence of minute quantities of chemicals that carry over from one insect life stage to another (Corbet 1985). For example, Barron and Corbet (1999) determined that minute traces of the larval diet on the puparium surface of *Drosophila* (Diptera: Drosophilidae) effectively induced a behavioral modification in the responses of emerging adult flies to their larval resource. Because traces of the larval diet can be found in the body of the immature insect, within the pupal cuticle, and on the surface of the puparium (Corbet 1985), there is no way for researchers to prevent contaminants from the larval environment from being present in the environment of the early-emerging adults (Barron 2001). Therefore, I feel as though the chemical legacy hypothesis provides a more plausible explanation for any preferences that may result from my studies of the response of adult *C. macellaria* to its larval resource.

Cochliomyia macellaria

Insect species colonizing human remains vary depending upon climatic conditions, geographic location, and stage of decomposition. Blow flies, also referred to as bottle flies, (Diptera: Calliphoridae) are normally the first to lay their eggs on a body during the fresh stage of decomposition.

Dipteran species associated with myiasis can be typed into three different categories: “Obligate” parasites deposit their eggs on living flesh; “Facultative” dipterans include those species that normally breed in dead or decaying material, but may also invade living tissue on occasion; and “Accidental” parasites are those that act as scavengers (Harrison and Pearson 1968). *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae), is considered a facultative parasite, as it is commonly present as a secondary invader in myiasis cases, generally feeding on the edge or surface of a wound (Harrison and Pearson 1968).

Cochliomyia macellaria, the secondary screwworm, is one of the most common colonizers of human remains in the southern United States and tropics, thus making it a fly of great forensic importance. The secondary screwworm is of particular importance to the livestock industry due to large economic losses resulting from disease transmission and myiasis. Unlike the primary screwworm, *Cochliomyia hominivorax*, *C. macellaria* larvae do not feed on living tissue; rather, they colonize a wound and feed on necrotic tissue.

The adult secondary screwworm generally ranges from five to eight millimeters in length, and its body appears metallic greenish-blue. Three dark green longitudinal stripes are present on the dorsal side of the thorax, and large orange-red colored eyes cover most of the head. Adults lay eggs in a mass consisting of 50 to 200 eggs, each of which is about one millimeter in length. It is not uncommon for group oviposition to occur, which may result in eggs entirely covering a large section of a carcass. Under normal conditions, the eggs hatch into cream-colored larvae, which can reach up to 17

mm in length as mature third instars. These larvae feed on tissues, organs, and bodily fluids of a corpse until they reach maturity, during which time they disperse away from the corpse in order to find a place to pupate. During pupation, the larva develops within the hardened outer skin of the last instar larva to eventually emerge as an adult fly. Because of the dearth of information regarding the development of *C. macellaria* and the relationship between blow flies and their attraction to larval resources, I examined the following objectives:

Objective 1: To examine the development of *C. macellaria* on equine and porcine muscle tissue at three different temperatures.

My hypotheses are:

H_{1o}: *Cochliomyia macellaria* larval development and adult survivorship are independent of larval resource and temperature.

H_{1a}: *Cochliomyia macellaria* larval development and adult survivorship are dependent on larval resource and temperature.

Objective 2: To examine the level of attraction of adult male and female *C. macellaria* to their larval resource.

My hypotheses are:

H_{2o}: Attraction of adult male and female *C. macellaria* to a resource is independent of what they fed on as larvae.

H_{2a}: Attraction of adult male and female *C. macellaria* to a resource is dependent on what they fed on as larvae.

Objective 3: To examine the attraction of adult male and female *C. macellaria* to resources with and without conspecific larvae.

H3o: Attraction of adult male and female *C. macellaria* to a resource is independent of the presence of conspecific larvae.

H3a: Attraction of adult male and female *C. macellaria* to a resource is dependent on the presence of conspecific larvae.

CHAPTER II

DEVELOPMENT OF *Cochliomyia macellaria* ON EQUINE AND PORCINE STRIATED MUSCLE TISSUE AT 21°C, 24°C, AND 27°C

Synopsis

Cochliomyia macellaria is commonly utilized in estimating the period of insect activity (PIA) on a corpse due to its rapid colonization time of fresh remains, active oviposition during daylight, and its abundance throughout the southern United States. Furthermore, *C. macellaria* is a secondary myiasis producer and is of great importance in human and veterinary forensic investigations of abuse and neglect. *C. macellaria* were reared on either equine or porcine striated muscle tissues at 21°C, 24°C, and 27°C. Cochliomyia macellaria needed approximately 35% more time to complete development when reared at 21°C instead of 27°C. Furthermore, larval growth and weight gain over time did not differ between larvae reared on equine versus porcine muscle tissue.

This study is the second in the United States to examine the development of *C. macellaria* and is the first to examine development of this species on muscle tissue from different vertebrate species. These data could provide significant information for myiasis and neglect cases of humans and animals.

Overview

Forensic entomology is the utilization of insects and other arthropods as evidence in both civil and criminal investigations (Williams and Villet 2006). The broad scope of

this field can be broken down into its three subdivisions which are urban, stored-product, and medicolegal entomology (Archer 2007). One important aspect of medicolegal entomology involves necrophagous arthropods colonizing and feeding on living and deceased human and animal tissue. Insects found on human remains may be utilized to estimate the period of insect activity (PIA) (Amendt et al. 2007). Identifying the oldest immature insect specimen associated with the remains can reveal the time elapsed between initial insect colonization and the discovery of the decedent. Because insects can arrive relatively soon after death, this PIA may also coincide with the post mortem interval (PMI) estimate (Archer 2007). However, the term "PMI" must be used with caution, as insect colonization may be delayed if conditions restrict access to the body (Benecke 2004).

Myiasis, the infestation and subsequent feeding upon living tissue, organs, or bodily fluids of a vertebrate host by dipteran larvae, can be used as evidence in cases of suspected neglect or abuse (Anderson and Huitson 2004). Myiasis most commonly occurs in elderly or very young individuals or in pets and livestock when there is an open wound or fecal matter on the animal for an extended time period (Anderson and Huitson 2004).

Myiasis most commonly occurs in wild animals, but many cases have been documented in domestic animals as well (Anderson and Huitson 2004). A world-wide animal abuse database on PetAbuse.com has been developed that contains 12,981 documented cases of animal cruelty in the United States, 625 of which are from the state of Texas alone. Because the number of animal abuse cases is increasing over time,

development data sets for myiasis-causing flies can help law enforcement officials better predict the duration of colonization for such cases.

Forensic entomologists depend on growth data from laboratory studies to estimate PIA for blow flies (Diptera: Calliphoridae) on human remains (Tarone and Foran 2006). Although pre-existing data sets within a blow fly species demonstrate similar development times, all do vary (Tarone and Foran 2006). Such variation could be due to a number of factors, such as climatic conditions, environmental variation, and genetic variation. For example, Tarone and Foran (2006) found that environmental conditions greatly influenced the development of the blow fly *Lucilia sericata* (Meigen). Their findings demonstrate the need for multiple development data sets for each species of blow fly, across geographic regions, as well as for a standardization of laboratory rearing techniques.

Development data utilized by forensic entomologists in estimating PIA are obtained from studies of growth of larvae that are usually reared on beef liver (Clark et al. 2006). However, it has been demonstrated that the host species on which the larvae feed may significantly alter the larval growth rate (Clark et al. 2006). For example, Clark et al. (2006) compared the development of *L. sericata* on lung, liver and heart, from both cows and swine. Their study determined larvae grew significantly faster on swine compared to cow, as well as when reared on lung and heart compared to liver. For forensic entomologists, such results emphasize the need for development data sets across tissue types, as well as across various species; they also demonstrate the importance of

recording the location from which blow fly larvae are removed from a corpse at a crime scene (Clark et al. 2006).

Cochliomyia macellaria (Fabricius), the secondary screwworm, is considered a facultative parasite, as it is commonly present as a secondary invader in myiasis cases, generally feeding on the edge or surface of a wound (Harrison and Pearson 1968). *C. macellaria* is one of the most common colonizers of human remains in the southern United States and tropics, thus making it a fly of great forensic importance (Hall 1948). The secondary screwworm is of particular importance to the livestock industry due to large economic losses resulting from disease transmission and myiasis (Anderson and Huitson 2004). Unlike the primary screwworm, *Cochliomyia hominivorax* (Coquerel), *C. macellaria* larvae do not feed on living tissue; rather, they colonize a wound and feed on necrotic tissue. The recent increase in the number of cases in the United States that involve forensic entomology further demonstrate the need for more development data sets in order to enhance the accuracy of estimating minimum PMIs (Grassberger and Reiter 2001). Currently, the only available *C. macellaria* development data available in the United States is for specimens reared on lean pork in Florida (Byrd and Butler 1996)

The primary objective of my study was to examine the development of *C. macellaria* on equine, porcine, and canine striated muscle tissue at different temperatures. I hypothesize that the developmental rates for *C. macellaria* will not differ across tissue types. Furthermore, I hypothesize *C. macellaria* development will not differ across the temperatures investigated.

Materials and Methods

Adult Fly Colony

C. macellaria (F.) larvae were collected from decomposing animal carcasses in the vicinity of College Station, TX, USA in 2008. Larvae were fed fresh beef liver in a 136LLVL Percival® (Percival Scientific, Inc., Perry, IA) growth chamber at approximately 27°C with 16:8 [L:D] h and 75-80% RH (Byrd and Butler 1996). Emergent adult flies were housed in 30 cm^3 screen cages and provided a 50:50 sugar: powdered milk mixture and fresh water *ad libitum* (Byrd and Butler 1996).A 114 mL Ziploc® (SC Johnson Products, Racine, WI) container with approximately 50 g of fresh beef liver covered with a folded paper towel was placed in the center of the screen cage when adult flies were seven-days-old. Hourly observations for oviposition were made. Eggs deposited were collected, homogenized, weighed gravimetrically, and utilized in the study. Four hundred eggs were placed on each treatment replicate. Only F_1 generation eggs were used in this study.

Tissue Source

C. macellaria development was examined on striated muscle tissue excised from the hind quarters of three different animal species. Three equines, three porcines, and three canines were utilized in this development study. Equine tissue was supplied by the Texas Veterinary Medical Diagnostic Laboratory (TVDML, Texas A&M University, College Station, TX). Porcine tissue was purchased from a local grocery. Canine tissue was donated by Dr. Everett Simmons, DVM (Burnet Road Animal Hospital, Austin,

TX). The animals utilized were free of barbiturates or other materials used for euthanization. Approximately 200 g of tissue were collected from each of the nine animal subjects and placed into separate Ziploc sandwich bags, labeled, assigned a temperature treatment, and stored in a 0°C freezer until used. Each tissue sample was allowed to thaw at least 24 h to reach the temperature of their assigned growth chambers prior to initiating the experiment. Tissue samples were remoistened with distilled water as needed. Tissue acquisition protocols were approved by the Animal Welfare Assurance Program, Texas A&M University, College Station, TX.

Constant Temperature

Three stand-up growth chambers each with three shelves were used for this study. *C. macellaria* development was observed at 21°C, 24°C, and 27°C. An Onset® HOBO U12-006 data logger with an Onset TMC6-HD air, water, and soil temperature sensor (Onset Co., Pocasset, MA) was placed on each shelf in each of the growth chambers. Each data logger recorded temperature hourly throughout the duration of the study. Temperature data during the initial run of the experiment indicate the 21°C chamber had a mean temperature of 20.84 ±1.25°C; the 24°C chamber's mean temperature was 24.35 ±0 .74°C, and the 27°C chamber had a mean temperature of 28.26 ± 1.08°C. Three replicates were conducted during a second experiment in October and the mean temperatures did not significantly differ.

Experimental Design

A Latin square design was used with three tissue types randomly assigned to each shelf within each of the growth chambers. A single replicate of each treatment was blocked on each shelf. Approximately 200 g of each individual animal subject was placed in a 1.10 liter styrene BioQuip® (BioQuip Products, Rancho Dominguez, CA) mosquito-breeding container. Each styrene container was placed in a Sterilite® (Sterilite Corporation, Townsend, WI) shoebox container. The Sterilite container was lined with 500 ml sand which served to restrict wandering larvae from escaping the container.

Life-History Traits

Hourly observations were made until egg hatch. After initial eclosion, three larvae were sampled from the respective containers every 12 h until pupae were observed. Each larva sampled was parboiled in water with weight, length, and instar recorded. Weights were taken using an Adventure-Pro AV64 Ohaus ® scale (Ohaus Corporation, Pine Brook, NJ). Lengths were recorded using a Meiji Techno ® EMZ-8TR microscope (Meiji Techno America, Santa Clara, CA).

Pupae were individually placed in 35 ml Jetware® (Jetware, Hatfield, PA) plastic medicine cups containing approximately 10 ml sand, capped with breathable lids, labeled, returned to the appropriate growth chamber, and monitored for adult emergence. Time to adult emergence and sex of individual were recorded. Adults dying during eclosion were not used in adult longevity analysis. Furthermore, unequal sample sizes per replicate due to failure of some adults to emerge from the pupal stage resulted in

development data not being analyzed by sex; however, overall sex ratio is provided. Resulting adults were monitored every 12 h for mortality. Adult flies were provided 0.20 ml distilled water via a 1cc Kendall Monoject ® SoftPack Insulin Syringe inserted through the lid every 24 h (Kendall, Mansfield, MA). Sex of each fly was recorded after death. Representative samples of the adult specimens obtained from this study were deposited in the Texas A&M University Arthropod Collection.

Statistical Analysis

All statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL). Analysis of covariance (ANCOVA) was used to compare development data for flies reared on each tissue type across temperatures. A Least Significant Difference (LSD) was performed to separate means following a significant F test ($P < 0.05$). The canine treatment was excluded due to insufficient replicates.

Results

Mean weight and length data for larvae reared on equine and porcine tissue are presented in Figures 1 and 2, respectively. Ranked weight and length data met basic assumptions of ANCOVA ($P < 0.05$). Intercept for larval weight gain did not significantly differ between tissue types at 21°C (df = 1, 65; $F = 0.157$; $P = 0.693$), 24°C (df = 1, 54; $F = 1.005$; $P = 0.321$), or 27°C (df = 1, 41; $F = 1.380$; $P = 0.247$) (Figure 3). However, hours elapsed significantly predicted larval weight at 21°C (slope = 0.993; t = 0.993; $P < 0.0001$), 24°C (slope = 1.214; t = 12.224; $P < 0.0001$), and 27°C (slope =

1.708; t = 12.805; $P < 0.0001$). Hours elapsed also explained a significant proportion of variance in larval weight for 21°C ($R^2 = 0.875$; df = 3, 65; $P < 0.0001$), 24°C ($R^2 = 0.863$; df = 3, 54; $P < 0.0001$), and 27°C ($R^2 = 0.880$; df = 3, 41; $P < 0.0001$). Hours elapsed significantly predicted larval length at 21°C (slope = 1.020; t = 14.529; $P < 0.0001$), 24°C (slope = 1.225; t = 11.808; $P < 0.0001$), and 27°C (slope = 1.735; t = 10.990; $P < 0.0001$). Hours elapsed also explained a significant proportion of variance in larval length for 21°C ($R^2 = 0.868$; df = 3, 65; $P < 0.0001$), 24°C ($R^2 = 0.850$; df = 3, 54; $P < 0.0001$), and 27°C ($R^2 = 0.838$; df = 3, 41; $P < 0.001$).

Intercept for larval length by hour did not significantly differ between tissue types at 21°C (df = 1, 65; $F = 0.505$; $P = 0.480$), 24°C (df = 1, 54; $F = 0.742$; $P = 0.393$), or 27°C (df = 1, 41; $F = 0.912$; $P = 0.345$) (Figure 4). However, based on a pairwise comparison, mean weight significantly ($P < 0.05$) differed for larvae reared on equine or porcine tissue at 21°C (df = 1, 65; $F = 6.957$; $P = 0.01$), 24°C, and 27°C. Therefore, tissue was removed from the analysis and ranked mean data for weight (Figure 5) and length (Figure 6) were analyzed with ANCOVA with temperature as the independent variable and hour as the covariate. However, temperature and hour interacted for length (df = 2, 166; $F = 16.03$; $P < 0.0001$) and weight (df = 2, 166; $F = 16.19$; $P < 0.0001$) thus violating the assumption that the independent and covariate do not interact. Therefore, the ANCOVA was not performed.

Degree day data for *Cochliomyia macellaria* development reared on equine and porcine tissue are presented in Table 1 using a base temperature of 10°C. Adults reared on porcine needed similar amounts of time to complete each stage of development as

they did when reared on equine tissue (Tables 1 and 2). However, development did differ for adults reared at different temperatures. Adults reared at 27°C needed approximately 31% and 21% less time to complete development than those reared at 21°C and 24°C, respectively. Sex rations were relatively equal (Table 2). Adult longevity (Table 2) was similar across tissue treatments; however those reared at cooler temperatures, 21°C and 24°C, lived longer than those reared at 27°C.

Discussion

Forensic entomology is the use of insects and other arthropods in legal investigations (Amendt et al. 2007). Such insect evidence is most commonly utilized in estimating the time of death, or postmortem interval (PMI) of a human corpse (Ames and Turner 2003) or more accurately the period of insect activity (PIA) on the corpse (Amendt et al. 2007). In order to estimate the PIA for arthropods collected from a set of remains, forensic entomologists rely on published laboratory growth data of these arthropods (Tarone and Foran 2006). Insects develop through a predictable species-specific life cycle at a temperature-dependent rate; therefore, if the temperature, species, and stage of insect are known, a forensic entomologist can determine amount of time the insects have been on the body (Anderson & Huitson 2004). By using such growth data to estimate the time of initial colonization, forensic entomologists are able to extrapolate a minimum PMI, or time since death of the deceased (Catts and Goff 1992). Data for egg eclosion, larval stage durations, pupal duration, and adult longevity times of

Cochliomyia macellaria, which commonly colonizes human cadavers in the southern United States, were collected in this study.

In addition to their use in estimating the PIA, blow fly development data sets can be used to determine a period of neglect (Lord 1990). Neglect cases most commonly occur in the very young or the elderly, as such individuals may possess open wounds or excrement on their bodies that act as potential targets for the same flies that live and feed on corpses (Benecke et al. 2004). Furthermore, increased awareness of malpractice of caregivers of the elderly has occurred in recent years due to there being more elderly individuals in our society than in past years (Benecke et al. 2004). Maggot fauna has even been utilized to illustrate neglect that occurred prior to the death (Benecke and Lessig 2001).

Data from this study can also be applied in cases of animal abuse and neglect. Myiasis may occur in animal abuse or neglect cases, which involve the infestation and subsequent feeding on living animals by fly larvae (Anderson & Huitson 2004). In most cases of myiasis, matted hair and the consequent buildup of urine and feces in the coat lead to flies laying their eggs in such areas (James 1947). In other cases, wounds may be present that initiate colonization (Anderson & Huitson 2004). By analyzing the fly larvae present in such cases, forensic entomologists are able to estimate a minimum length of time that the neglect or abuse has been taking place.

Although canines are the species most commonly victimized by myiasis (Anderson & Huitson 2004), cats, rabbits, sheep, and horses may also experience this type of infestation. Because the horse industry is so important in the southern United

States, a development data set for *C. macellaria* on equine tissue was conducted. These data indicate that development on muscle tissue from different species does not differ for *C. macellaria*. Consequently, this data set could be applied in cases regarding colonization of porcine, equine, and potentially other vertebrate species. Prior to this study only one other development data set exists for *C. macellaria* in the United States, which was performed on blow fly populations in Florida (Byrd and Butler 1996). Byrd and Butler's study (1996) assessed the development of *C. macellaria* on porcine tissue across five temperature regimes. Their study encompassed two temperatures of 21.1°C and 26.7°C, which should result in mean stage durations that are comparable to those in this study which were performed at 21°C and 27°C; however, the mean time it took specimens in this study to complete each stage of development was greater than Byrd and Butler's resulting mean stage durations. The only exceptions to this were for the egg stage at 27°C and the 1st instar stage at 21°C, both of which took less time to complete for my study. A number of factors could explain the greater development times that resulted from my study, when compared to Byrd and Butler (1996).One factor accounting for mean differences in development time is that Byrd and Butler (1996) utilized cyclic temperature regimes for their 21.1°C and 26.7°C temperatures, each with an amplitude of 5.5°C while temperatures in the study were stable.

Genetic variation across populations could provide an explanation of the variation between this study and Byrd and Butler (1996). For example, several authors have generated development data tables for different fly populations of the blow fly *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) which were exposed to various

environmental conditions. The resulting developmental times differed from one another, as Kamal (1958), Greenberg (1991), and Grassberger and Reiter (2001) estimated faster minimum stage durations than Anderson (2000). In order to assess this issue of varying development times, Tarone and Foran (2006) preformed a study that sought to estimate variation in developmental rates resulting from genetic differences in a single population of *L. sericata*. Tarone and Foran (2006) reared specimens under laboratory conditions mimicking those used in the previous studies. Given the minimum developmental times of their resulting treatments, the fastest development time for this species fell within the standard errors presented by Greenberg (1991) and Grassberger and Reiter (2001); similarly, the slowest minimum developmental duration times for flies in this study were longer than the minimum developmental times generated by Anderson (2000). Tarone and Foran (2006) concluded that genetic variation alone can potentially explain the differences in developmental times generated in the other studies.

Environmental conditions within a region for select fly populations can successfully propagate their resulting genetic variation. Resulting genetic variation among blow fly populations across the regions studied previously could also explain the variation observed between this study and Byrd and Butler (1996). For example, Ames and Turner (2003) determined that it is possible for the same species from different geographical locations to have different thermal constant accumulated degree hour (ADH) values. They reached this conclusion based on their study of the effects of low temperature episodes on the larval development of *Calliphora vicina* (Robineau-Desvoidy) (Diptera: Calliphoridae) and *Calliphora vomitoria* (Linnaeus) (Diptera:

Calliphoridae). Although the effects of genetic makeup of *C. macellaria* populations have been largely untested, genetic variability due to different environmental conditions may be important sources of developmental variability when comparing different populations (Tarone and Foran 2006).

Laboratory protocols for rearing *C. macellaria* might explain the biological variation observed. Such factors may include the difference in handling methods during rearing, as well as the use of different rearing chambers. Even if the variation in development times is not actually attributed to differences in laboratory rearing protocols, the current study reemphasizes the critical importance of establishing a common set of rearing conditions, which best mimic growth on actual carrion, in order to compare blow fly data sets and use them in legal investigations (Tarone and Foran 2006). If such standardized protocols were developed by forensic entomologists, researchers could then focus their work on the variability between populations that can be attributed to genetic variability.

Variability between similar tissue type but different carrion species must be assessed in order to explain development variation that might exist, and therefore allow for more accurate PIA estimates. My study assessed the development of *C. macellaria* on equine and porcine striated muscle tissue. Although no significant difference in mean larval length or weight existed between similar tissues from these two species, Clark et al. (2006) determined that the species origin on which larvae were reared may result in significantly different mean development times. Clark et al. (2006) did not utilize striated muscle tissue in their study; however, their study determined that larvae grew

significantly larger on porcine liver compared to bovine liver, with an overall difference in the detransformed mean lengths being 0.63 mm. These results reveal the importance for future studies to address the possibility that further differences in larval growth rates may occur between animal and human tissues (Clark et al. 2006).

Variability between different body tissues within the same animal species was also assessed by Clark et al. (2006) in order to explain development variation that might exist. Clark et al. (2006) found that *L. sericata* larvae developing on porcine liver took approximately 31 more hours to complete the feeding stage and grew an average of 2 mm shorter than larvae reared on lung of the same species. *Calliphora vicina* also experienced variations in larval stage durations, depending upon which type of tissue they were reared (Kaneshraja and Turner 2004). *C. vicina* completed larval feeding stages up to two days faster when fed pig lung, heart, kidney or brain as compared to pig liver (Kaneshraja and Turner 2004). Most research on the development of forensically-important blow flies has focused on gathering data for the egg (Anderson and Huitson 2004) or larval stages (Grassberger and Reiter 2001, Greenberg 1991, Tarone and Foran 2006). Although some data on adult longevity for such blow flies do exist (Gabre et al. 2005), this study is the first to break down adult longevity based on sex. These results indicate that males and females each live for comparable amounts of time after emergence; this information could be useful in abuse or death investigations where empty pupal cases are found in the area of a corpse, thus indicating that the flies have been given enough time to complete their entire life cycles. Subsequently, living adult blow flies could be collected from the death scene and maintained under the conditions

used in this and time of death recorded. This information could provide insight to time since emergence and consequently a more refined estimate of the time of colonization. Similarly, dead adult flies collected from a scene could be analyzed based on the degree day model (Table 1), as they may indicate that the adults have completed their minimum longevity durations.

CHAPTER III

ADULT PREFERENCE OF *Cochliomyia macellaria* IN RESPONSE TO LARVAL RESOURCE AND THE PRESENCE OF CONSPECIFIC LARVAE

Synopsis

The goal of this study was to determine whether or not adult male and female *C. macellaria* blow flies demonstrated a preference for their larval resource based on olfactory responses in a Y-tube olfactomber. Flies were reared either on beef liver or testicles. Initial and final responses as well as residence time in each arm of the olfactometer over a five minute period were recorded. An additional experiment for flies reared on liver was conducted examining adult male and female preference for resources with and without conspecific larvae present. Seven to ten day old blow flies were used in these experiments. Variables previously listed were recorded for this study. Only final choice of females reared on liver demonstrated a significant ($\alpha = 0.05$) attraction for larval resource on which they were reared. All flies reared on beef testicles showed a preference for their larval resource in initial and final choices and residence time. Based on chi-square analysis, no significant difference was determined for the parameters measured for either sex in response to the presence of conspecific larvae. Data indicate that adult male and female *C. macellaria* flies are generalist and do not show a preference for the larval resource on which they were reared or for resources inoculated with conspecific larvae. However, residence time does appear to provide a

more clear indication of the responses of *C. macellaria* in the Y-tube than initial and final choice.

Overview

Learning can be described as any modification in behavior due to experience (McCall and Kelly 2002). The ability to learn is an essential mental function which depends upon an organism's ability to perceive information. The ability to acquire, store, and retrieve information from an animal's experiences is genetically based, but the information is acquired within that animal's life (McCall and Kelly 2002).

The capacity to learn is universal among animals (Hendel et al. 2005). Because animals live in a world of dynamic stimulus events, innate behavioral responses to such events are imperative to that animal's survival (Daly and Smith 2000). Under many circumstances, there are advantages for the animal's responses to display some degree of plasticity based on prior experience (Daly and Smith 2000). For insects, such advantages include the ability to optimize foraging, evade predators, find potential mates, and locate hosts (Daly et al. 2001)).

Associative learning has been extensively studied in a wide range of arthropods, including moths (Lepidoptera: Sphingidae), bees (Hymenoptera: Apidae), and mosquitoes (Diptera: Culicidae), (Daly et al. 2001, McCabe et al. 2007, Tomberlin et al. 2006). However, few have undertaken studies that seek to assess the ability of forensically-important arthropods to learn. Because blow flies (Diptera: Calliphoridae) are generally the first to arrive at human and animal remains, understanding their ability

to memorize and learn would undoubtedly prove to be beneficial in the field of forensic entomology. Although a variety of associative learning research studies involving blow flies are currently underway, a few publications have paved the way for such studies (McGuire 1981, Nelson 1971, Dethier 1992).

One research experiment assessed the central excitatory state (CES) response in the blow fly, *Phormia regina* (McGuire 1981). The CES is a change in the central nervous system of a fly that occurs after sugar stimulation so that the fly would respond to some inappropriate stimulus by extending its proboscis (McGuire 1981). It has been suggested that prolonged mounting or immobilization of flies may also induce the CES response, and that such a response is involved in the conditioning of blow flies (Nelson 1971). In short, Nelson implied that classical conditioning may be an extension of the CES response, although her evidence was inconclusive.

The "learned response" of proboscis extension may be due to the CES alone under conditions of stressful or prolonged mounting, or the CES may be a predisposition for learning (McGuire 1981). McGuire performed a study which assessed the relationship between classical conditioning and the CES in the blow fly, *Phormia regina*. Response to selection for CES was very fast in his experiments, suggesting that behavioral differences in CES are correlated with differences at a single locus (McGuire 1981). McGuire commented that CES selection experiments are a first step in the analysis of classical conditioning in blow flies.

Dethier performed experiments that assessed behavioral habituation in *Phormia regina* (Dethier 1992). Behavioral habituation involves the ceasing of an animal's

responsiveness to a stimulus (Dethier 1992). His study involved releasing wingless blow flies onto a flat uniform substrate and analyzing their hopping movements, as hopping is an action commonly associated with flight initiation. He noticed that the rate of hopping decreased over time, resembling behavioral habituation. Dethier was able to reestablish hopping of his *P. regina* subjects through tactile stimulation. His study sought to assess the relationship between habituation and physiological variables, such as circadian rhythms or age of the test subject; neither of these variables were shown to attribute to the waning of hopping in the flies (Dethier 1992).

The assumption that adult insects prefer the resource on which they themselves developed as larvae is known as the Hopkins' host-selection principle (HHSP) (Hopkins 1916). HHSP implies that insects experience what is referred to as preimaginal conditioning, meaning that the behavior of an adult insect is conditioned by larval experience (Barron 2001). This principle was widely accepted at the time of its initial proposal in 1916, but has been condemned by researchers in recent years due to the numerous factors that contribute to a behavioral bias for the developmental host insect (Barron 2001). Inducing a food resource or host preferences in adult insects by exposing them as larvae to a specific food source or host has been performed in early demonstrations of preimaginal conditioning (Thorpe 1939, Manning 1967); however, convincing results from such demonstrations in holomotabolous insects are rare (Barron 2001). Because such studies have been received with skepticism for failing to effectively isolate emergent adult insects from their preimaginal environment (Jaenike 1998), the proposal that adults respond to a "chemical legacy" of their larval environment has

become accepted as the reason for adult insect behavior in response to their larval resource (Corbet 1985).

The chemical legacy theory implies that an adult behavioral change is acquired by insects in the early-emergent stage, rather than preimaginal stage (Barron 2001). This legacy takes into account the persistence of minute quantities of chemicals that carry over from one insect life stage to another (Corbet 1985). For example, Barron and Corbet (1999) determined that minute traces of the larval diet on the puparium surface of *Drosophila* (Diptera: Drosophilidae) effectively induced a behavioral modification in the responses of emerging adult flies to their larval resource. Because traces of the larval diet can be found in the body of the immature insect within the pupal cuticle, and on the surface of the puparium (Corbet 1985), there is no way for researchers to prevent contaminants from the larval environment from being present in the environment of the early-emerging adults (Barron 2001). Therefore, I feel as though the chemical legacy hypothesis provides the soundest explanation for any preferences that may result from my studies of the response of adult *C. macellaria* to its larval resource.

A variety of insects are capable of learning. However, an extremely limited number of publications have assessed the concept of learning and memory in forensically-important arthropods, such as members of the family Calliphoridae. By understanding how calliphorid adults effectively locate and exploit certain oviposition sites, such as human corpses, forensic entomologists would be better able to assess the circumstances surrounding the period of insect activity (PIA) in death investigations. Through the implication of associative learning studies of blow flies, forensic

entomologists will be able to better interpret why blow flies colonize certain carrion over others, what volatiles attract the flies to carrion, and what factors affect the choice to oviposit (such as the presence of a predatory species); understanding what associations are linked to such behaviors will inevitably lead to more accurate estimates of PIA, as well as give researchers insight into the mental capacities of these important insects.

Cochliomyia macellaria (F.) (Diptera: Calliphoridae) is one of the first blow flies to arrive at a corpse during warm months in the southern United States. Consequently, understanding its biology is critical to accurately predicting the time since death. My research involved two studies of associative learning that asked the following questions: 1) Are adult *C. macellaria* flies more attracted to their larval resource? and 2) Does the presence of conspecific larvae affect attractiveness of a resource to adult *C. macellaria* flies?

Materials and Methods

Adult Fly Colony

C. macellaria (F.) larvae were collected from decomposing animal carcasses in the vicinity of College Station, TX, USA in 2008. Larvae were fed fresh beef liver in a 136LLVL Percival® (Percival Scientific, Inc., Perry, IA) growth chamber at approximately 27°C with 16:8 [L:D] h and 75-80% RH (Byrd and Butler 1996). Emergent adult flies were housed in 30 cm^3 screen cages and provided a 50:50 sugar and powdered milk mixture and fresh water *ad libitum* (Byrd and Butler 1996). A 114 ml Ziploc® (SC Johnson Products, Racine, WI) container with approximately 50 g of fresh

beef liver covered with a folded paper towel was placed in the center of the screen cage when adult flies were seven-days-old. Hourly observations for oviposition were made. Eggs deposited on the paper towel were collected, homogenized, weighed gravimetrically, and utilized in the study.

Conditioning to Larval Resource

Eggs were collected less than one hour after oviposition, homogenized in distilled water, and equally partitioned between two treatments. Approximately 200 larvae were reared either on beef liver or testicles. Each resource inoculated with blow fly eggs was kept in an individual growth chamber set at 27°C with 16:8 [L:D] h and 75-80% RH. The assigned resource was added *ad libitum*. Containers were examined daily for pupae, which were and placed in 1.101 liter styrene BioQuip® (BioQuip Products, Rancho Dominguez, CA) mosquito-breeding containers located in 30 cm^3 mesh cages in the assigned growth chamber. Resultant adults were provided a 50:50 mixture of powdered milk and sugar, and distilled water *ad libitum*. Attraction of adults at least 7 to 12d-old to their respective larval resource was examined (Thomas 1993).

Y-Tube Olfactometer

A Teflon® dual-choice olfactometer (Figure 1) was used to assess associative learning of *C. macellaria* to respective larval resources. The stem of the olfactometer measured 13.97 cm with each arm measuring 17.78 cm. The width and depth of the olfactometer stem was 7.62 cm x 5.08 cm, respectively, and the width and depth of the

arms were 6.35 cm by 5.08 cm, respectively. The olfactometer was covered with a sealed 0.5 cm thick sheet of glass and had a small fan that produced 0.1 liter/s airflow from the base of the stem.

Flies were inserted into the olfactometer through a 1.3 cm hole located on the floor of the stem approximately 3.81 cm from the base of the olfactometer stem. The olfactometer was engaged when each fly was introduced into the olfactometer. The entry was then plugged with a rubber stopper. Initial choice (i.e. which arm entered) and final choice (fly location at conclusion of 5 min observation period) were recorded. Chi square analyses of data results indicate initial and final choices (Figures 1 and 2) are random ($P > 0.05$). Time spent in each arm during the 5 min interval was also recorded. Therefore the amount of time the fly spent in each arm during the 5 min interval was analyzed. The fly was removed and preserved in 80% ethanol at the conclusion of the 5 min interval.

Treatments

The base of each arm was connected to a 6.35 cm by 1.11 cm Nalgene Labware® tube (Thermo Fisher Scientific Rochester, NY). Each tube was inserted into the side of a 114 ml Ziploc® (SC Johnson Products, Racine, WI) container with either 100 g of beef testicle or liver. Both containers were covered with an airtight lid. The side opposite of the plastic tubing had a 1.5 cm hole which was capped with a charcoal filter.

Male and female flies reared on each resource were used in this experiment. Ten testicle-fed males, ten liver-fed males, ten testicle-fed females, and ten liver-fed females

were examined between 0700 and 1400 hr daily for three consecutive days. Flies were collected from each treatment colony and placed in a 15 ml glass vial and allowed to acclimate a minimum of 5 min before testing. Each fly was tested only once. Individuals not responding were discarded and another fly from the treatment colony tested. The glass top and olfactometer were cleaned with 80% ethanol and air dried for one minute between each test. Additionally, the containers with the resources and plastic tubing were alternated between each arm.

Conspecific Larvae

A separate experiment was performed that assessed attraction of adult *C. macellaria* flies to 50 g of liver with 60 third instar conspecific larvae verses 50 g of liver without the presence of larvae. Five adult male flies and five adult female flies were tested in the olfactometer between 0700 and 1400 hr daily for four consecutive days. All adults used in this experiment were fed beef liver as larvae and kept under the same rearing conditions as used in the larval resource study. The treatments used for this experiment were 50 g of liver with 60 third instar conspecific larvae at the end of one arm of the olfactometer and 50 g of liver without larvae at the end of the other arm. All olfactometer settings and testing methods were the same as in the larval resource objective.

Statistical Analysis

A chi-square analysis was performed with SPSS (SPSS Inc., Chicago, IL) for the initial and final responses for each sex to determine if flies exhibited a significant ($P < 0.05$) preference for each treatment. Time spent in each arm was transformed into categorical data. The arm with greatest residence time of a fly was assigned a one while the other arm was assigned a zero.

Results

All flies reared on beef liver showed a preference for their larval resource in initial choice and residence time (Figure 1). Analysis of final choice differed between male and female. Males showed a preference for their non-larval resource, while females preferred their larval resource. Only female final choice was significant ($\alpha = 0.05$, $\chi^2 = 43.77$; df = 29; $P = 0.0214$) for larval resource. All flies reared on beef testicles showed a preference for their larval resource in initial and final choices and residence time (Figure 2). Based on chi-square analysis, only male initial choice was significant ($\alpha = 0.05$, $\chi^2 = 43.77$; df = 29; $P = 0.0494$) for larval resource.

Initial and final choices and residence time were recorded for male and female to beef liver with and without third instar conspecific larvae. In the case of males, they showed a preference for the resource without larvae when recording final choice and residence time (Figure 3). Male initial response indicated a preference for liver with larvae. In contrast, females showed a preference for liver with conspecific larvae for initial and final choices and residence time. However, based on chi-square analysis, no

significant ($\alpha = 0.05$) difference was determined for the parameters measured for either sex.

Discussion

Cochliomyia macellaria is one of the primary consumers of carrion (Braack 1986). In most instances carrion often receives more eggs from colonizing insects or larvae than it can support (Kneidel 1984). Blow flies are rarely able to complete more than one generation on a single carrion item (Ives 1988). Carrion flies, such as Calliphoridae and Sarcophagidae, possess natural histories that are thought to involve intense competition for larval food resources (Wells and Greenberg 1994). High selection for rapid location and consumption of patchy and ephemeral carrion resources is evident in almost every aspect of the breeding biology of blow flies (Hanski 1986).

There are obvious advantages and drawbacks to being either a specialist species or a generalist species of insect fauna. Hanski (1976) defines "generalists" as species that are capable of exploiting a range of food resources wider than animal carcasses. True carrion specialists require specific suitable conditions for successful exploitation of a given resource, in which case only a few species may effectively be able to decompose all of the available carrion (Hanski 1976); this implies that specialist species may encounter less competition from other species, but run into the issue of having to exploit only specific types of carrion. In cases where the specialist fauna is "incomplete," or not present, more generalist species are given the opportunity to colonize the carrion (Hanski 1976). Generalist blow fly species have the ability to exploit a wide range of carrion, but

are likely to experience increased competition from other species. Therefore, due to the ephemeral and patchy distribution of carrion, it is essential that adult blow flies be able to detect and locate such resources as quickly as possible in order to increase the likelihood of survival of their offspring.

It has been suggested that female Calliphoridae are strongly selected to oviposit in safe, nutritious locations that are protected from the elements, predators, and parasitoids (Archer and Elgar 2003b). Areas of patchy resources that are at a lower risk from predators, desiccation and competition are exploited by larvae of carrion-breeding blow flies (Archer and Elgar 2003b). Such areas often include orifices and body folds of carrion, which provide protection and a readily-available food source (Archer and Elgar 2003b). Archer and Elgar (2003b) investigated the preferences of adult female carrion flies and found that these insects initially preferred to oviposit in the mouth of carrion but, after 24 hours of exposure to the resource, began to deposit offspring in body folds.

An incomplete understanding of the factors that attract forensically-important adult calliphorids to carrion exists (Archer and Elgar 2003a). However, studies have demonstrated that different resource requirements motivate males, gravid females and non-gravid female blow flies to exploit carrion (Spradberry 1979). Certain circumstances may require forensic entomologists to make assumptions about the time of oviposition by female blow flies, so more insight into the factors governing blow fly attraction to carrion is essential (Archer and Elgar 2003a). Archer and Elgar (2003a) found that gravid female blow flies may be present at a resource that is no longer suitable for oviposition. They also discovered that fewer flies were present at carrion that was in a

stage of advanced decomposition, as compared to remains that were fresh. These results suggest that a combination of altered chemical cues as well as a reduced volume of attractive chemicals that are emitted during later stages of decay could have significant impacts on the abundance of blow flies that are present at a corpse throughout the decomposition process (Archer and Elgar 2003b).

Some calliphorid species have been found to frequently mate on carcasses, which often leads to early development females representing the highest carcass attendance (Archer and Elgar 2003b). Archer and Elgar's (2003b) results indicate that male attendance at a carcass increases when they are more likely to encounter non-gravid females in the early stages of egg development. However, males are not found at carrion sites as often as female blow flies, possibly due to the findings that many calliphorid males set up "mating situations" at alternate sites, such as nectar and honeydew sources that attract females (Archer and Elgar 2003b). Evidence supports this theory that males are more common at carbohydrate than proteinaceous sources (Guillot et al 1977). However, it has been found that females of certain blow fly species release airborne sex pheromones once receptive, which may serve as attractants to males to carrion-breeding sites (Bartell et al 1969).

The original goal of my research was to assess the concept of associative learning in *C. macellaria* in response to larval resource, as well as the effect of presence of conspecific larvae on a food resource. However, after investigating other explanations for adult blow fly preference, I decided that blow flies experience what is referred to as chemical legacy, rather than associative learning. Once the larvae in my experiment

pupated, the outside of the pupal casings still possessed chemicals and compounds from the larval resource, either beef testicles or beef liver, depending upon the treatment. These persisting chemicals from the earlier larval stages may have a particularly significant impact on the insect's responsiveness during the "sensitive period" during which time the fly first emerges from the pupa (Corbet 1985). This phenomenon is referred to as the chemical legacy hypothesis, which proposes that effects of a blow fly's early environment on the chemosensory responsiveness of a later stage depend not on associative learning, but on the direct effects on the later stage itself of a legacy of chemical cues that were present in an earlier, immature stage (Corbet 1985). This theory implies that minute quantities of certain chemicals, such as those left on the pupal casings from the given larval resource, persist from one stage to another inside or outside the insect's body (Corbet 1985). The chemical legacy hypothesis provides a more sound explanation than associative learning for the behavior of adult carrion flies, such as *C. macellaria*, when choosing a resource to colonize. This theory offers forensic entomologists a non-genetic explanation for the origin and persistence of a heritable change in carrion preference and subsequent exploitation (Corbet 1985).

Until recently, the majority of forensic entomology research has focused on its application from time of colonization of remains to the time of discovery by law enforcement, which is referred to as the post-colonization interval (post-CI). However, a lack of attention has been given to the behavior of blow flies from the time of death to the initial colonization of remains, also known as the pre-colonization interval (pre-CI) (R. Mohr, *unpublished data*). The pre-CI involves cues that lead to the fly detecting and

locating a given resource. By discovering what types of cues are driving forensically-important blow flies to locate a resource to colonize, forensic entomologists will gain a better understanding of the biology of such flies and, thus, be able to develop more accurate minimum PMI estimations. Therefore, it is essential that future research focus on what types of cues are initially attracting forensically-important blow flies to carrion, whether they are chemical, olfactory, visual, or pheromonal. A potential starting point for discovering such ecological influences could be to analyze the compounds that are present on specific types of carrion, such as human cadavers. Research should also assess what compounds and chemicals are given off by larvae and eggs of other blow flies that may be present on carrion, as such olfactory cues may influence the oviposition of inter- and intraspecific adults on carrion that has already been colonized.

CHAPTER IV

CONCLUSION

Forensic entomologists can use the development data for *C. macellaria* from my thesis to estimate PIA on human remains. This study is the first in Texas and second in the United States to examine the development of *C. macellaria* and could provide significant information for cases of myiasis and neglect of both humans and animals. This study also provides the first data set of *C. macellaria* development on equine tissue at different temperatures. The utilization of equine tissue is important due to the recent increase in equine neglect cases in Texas, as well as the fact that the equine industry is so economically important to those living in Texas. This research is also landmark in that it assessed adult longevity based on sex, in addition to the egg, immature, prepupal, and pupal stage durations. The adult longevity data allows forensic entomologists to collect both live and dead adult *C. macellaria* flies from a crime scene and utilize them to develop a PIA; adult specimens have not been utilized in such a manner up until this point. Furthermore, because *C. macellaria* is a secondary myiasis producer, this development data will also be useful in veterinary forensic cases. This is important because animal abuse cases are on the rise and have been given special media attention in recent years. Overall, this study will also provide better insight on the development of *C. macellaria* in Texas as it relates to tissue type and temperature.

Until recently, the majority of forensic entomology research has focused on its application from time of colonization of remains to the time of discovery by law

enforcement, which is referred to as the post-colonization interval (post-CI). However, a lack of attention has been given to the behavior of blow flies from the time of death to the initial colonization of remains, also known as the pre-colonization interval (pre-CI). The pre-CI involves cues that lead to the fly detecting and locating a given resource. By discovering what types of cues are driving forensically-important blow flies to locate a resource to colonize, forensic entomologists will gain a better understanding of the biology of such flies and, thus, be able to develop more accurate minimum PMI estimations. My research involving adult *C. macellaria* attraction to larval resource and conspecific larvae represents the first of its kind for this species of blow fly. Although my initial hypothesis was that blow flies learn to associate their larval resource with a sufficient adult oviposition site, further examination into the mechanisms by which adult blow flies select certain resources reveals that these insects are probably experiencing a chemical legacy. This is supported by the fact that compounds of a blow fly's larval resource remains inside of the insect's body and on the outside of the puparium, thus allowing newly-emerged adults to be exposed to the compounds from their larval resource.

Although no significant difference in larval length, weight, or development time existed between porcine and equine tissue, it is important for future research to examine the development of blow flies across different species of tissue. Likewise, such research should also further assess variation that may occur in blow fly development across different body tissues within the same species of carrion. These concepts have important implications in forensics, as it has never been demonstrated that blow flies develop the at

the same rates as they have been demonstrated to do so on animal tissues that have been utilized in all current development studies. The need for multiple development data sets across the United States is it is essential to account for genetic variability within a fly species that may occur throughout different geographic regions.

Future research focus involving the types of cues that initially attract forensically-important blow flies to carrion, whether they be chemical, olfactory, visual, or pheromonal, must also be expanded. A potential starting point for discovering such ecological influences could be to analyze the compounds that are present on specific types of carrion, such as human cadavers. Research should also assess what compounds and chemicals are given off by larvae and eggs of other blow flies that may be present on carrion, as such olfactory cues may influence the oviposition of inter- and intraspecific adults on carrion that has already been colonized. By learning more about the biology of blow flies and by what mechanisms they are attracted to a resource, forensic entomologists will be able to better estimate the PIA in legal investigations.

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APPENDIX A

FIGURES

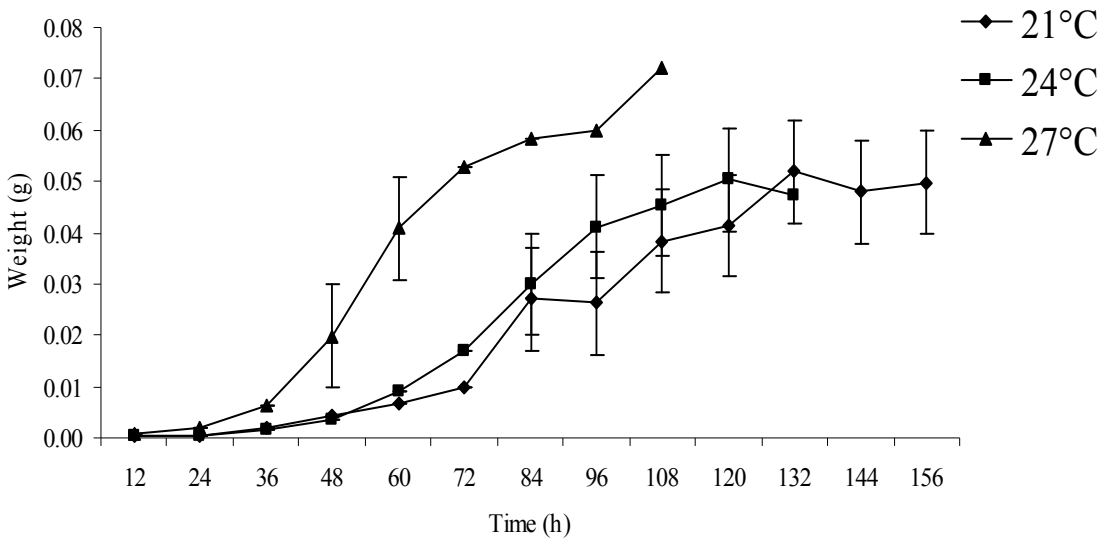

Figure 1. *Cochliomyia macellaria* larval weight (n = 3) ± SE developing at three temperatures over time.

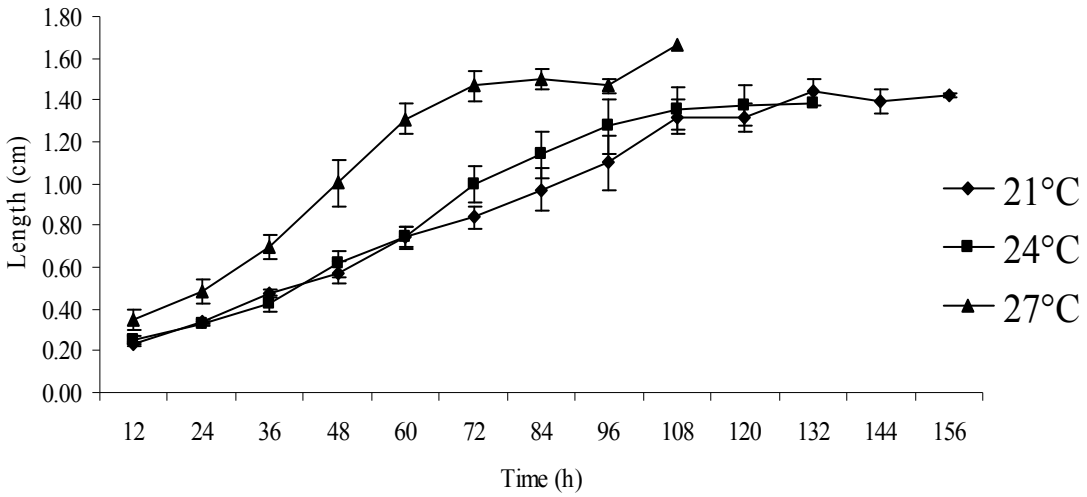

Figure 2. *Cochliomyia macellaria* larval length (n = 3) ± SE developing at three temperatures over time.

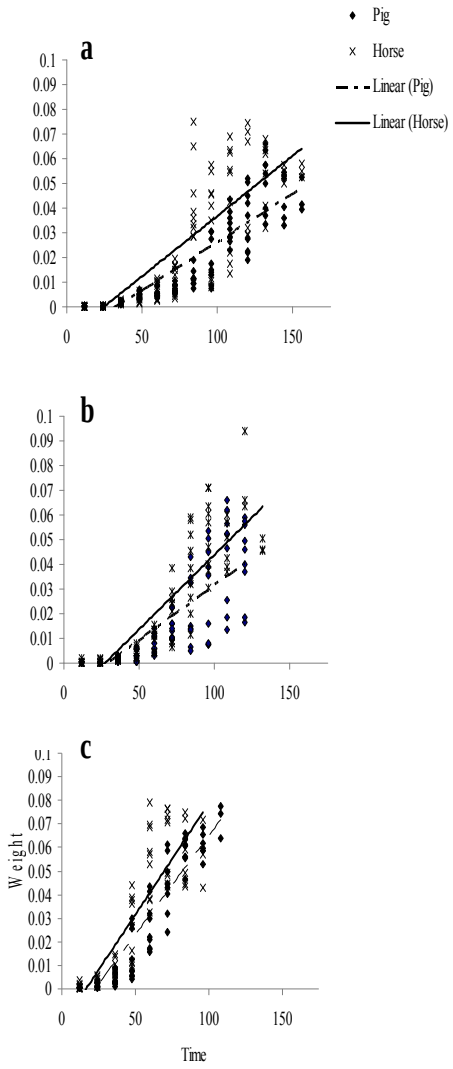

Figure 3. Ranked weight data using the calculated x-intercept and ANCOVA results for larvae reared at a) 21°C, b) 24°C, and c) 27°C.

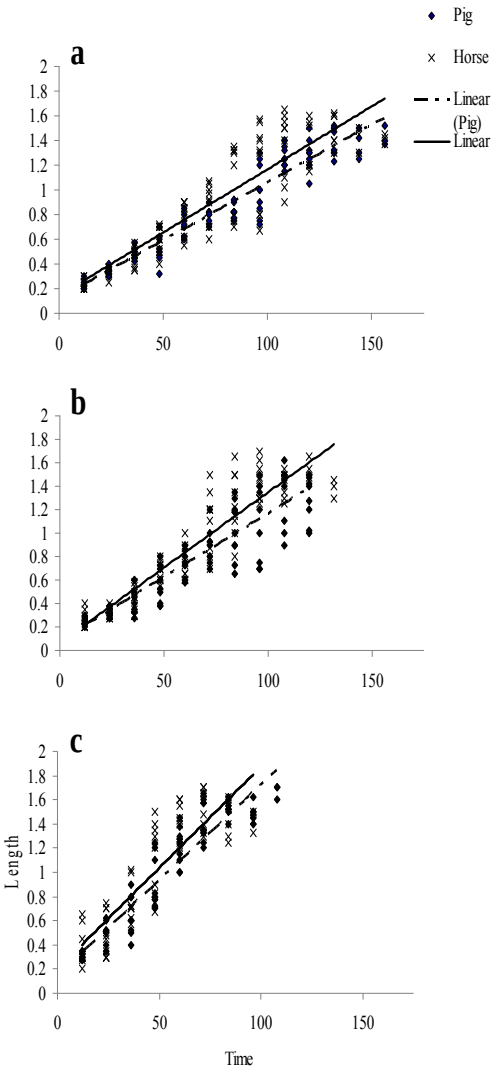

Figure 4. Ranked length data using the calculated y-intercept and ANCOVA results for larvae reared at a) 21°C, b) 24°C, and c) 27°C.

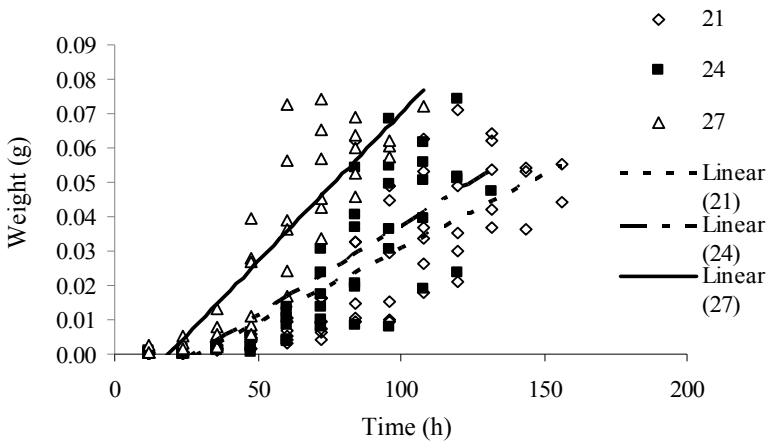

Figure 5. Ranked larval weight data with temperature (21°C, 24°C, and 27°C) as the treatment using the calculated x-intercept and ANCOVA results.

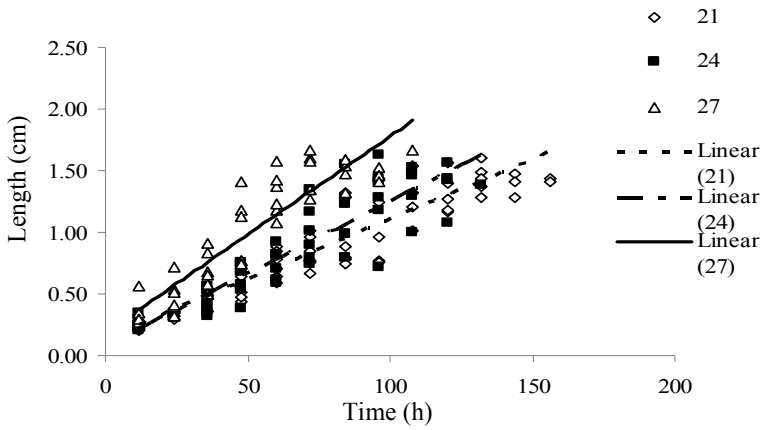

Figure 6. Ranked larval length data with temperature (21°C, 24°C, and 27°C) as the treatment using the calculated y-intercept and ANCOVA results.

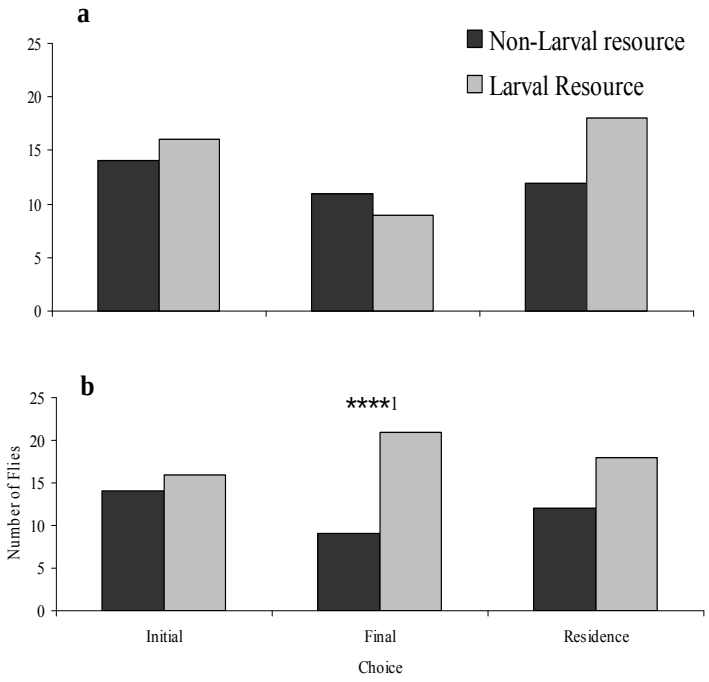

Figure 7. Initial and final response and residence time of F_1 7-12-d-old (a) male and (b) female *Cochliomyia macellaria* (n = 30) reared on beef liver and offered beef liver and testicles in a Y-tube olfactometer. [1]****χ^2 =43.77; df = 29; *P* = 0.0214

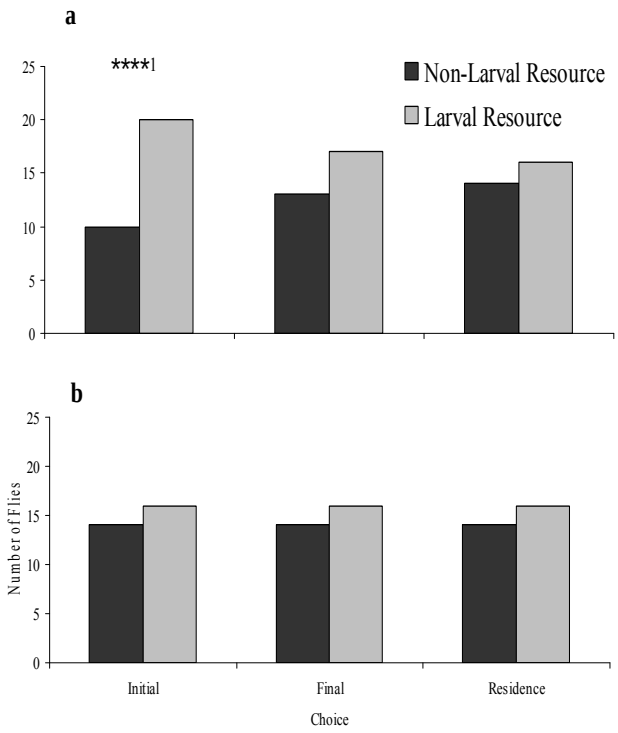

Figure 8. Initial and final response and residence time of F_1 7-12-d-old (a) male and (b) female *Cochliomyia macellaria* (n = 30) reared on beef testicles and offered beef liver and testicles in a Y-tube olfactometer. [1]****$\chi^2 = 43.77$; df = 29; $P = 0.0494$

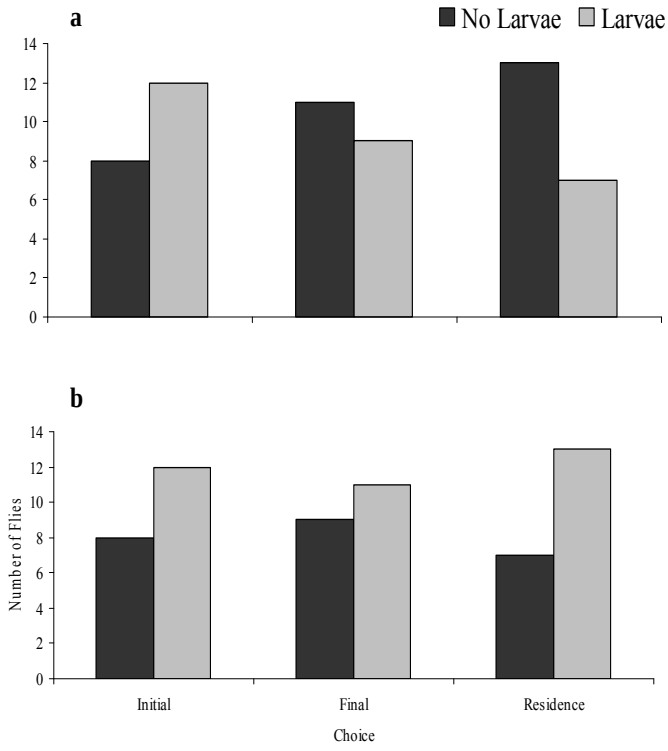

Figure 9. Initial and final response and residence time of F_1 7-12-d-old (a) male and (b) female *Cochliomyia macellaria* (n = 20) to liver without and liver with approximately 60 third instar conspecific larvae in a Y-tube olfactometer.

APPENDIX B

TABLES

Table 1. Minimum degree days and accumulated degree days for development of *Cochliomyia macellaria* (n = 3) on equine and porcine muscle at three temperatures[1].

Temperature	Tissue		Egg ± SE (DD[2])	First ± SE (DD)	Second ± SE (DD)	Third ± SE (DD)	Prepupal ± SE (DD)	Pupal ± SE (DD)
21								
	Equine		8.40 ± 0.40	11.00 ± 0.00	31.17 ± 3.67	60.50 ± 6.35	8.68 ± 0.56	70.56 ± 3.15
		Mean ADD[3]		19.40	50.57	111.07	119.75	190.31
	Porcine		8.10± 0.31	11.00 ± 0.00	31.17 ± 3.67	66.00 ± 3.18	7.22 ± 0.61	66.81 ± 0.49
		Mean ADD		19.10	50.27	116.27	123.49	190.30
24								
	Equine		10.69 ± 0.39	14.00 ± 0.00	37.33 ± 2.33	65.33 ± 6.17	9.45 ± 1.81[4]	64.02 ± 1.71
		Mean ADD		24.69	62.02	127.35	136.80	200.82
	Porcine		10.11 ± 0.19	14.00 ± 0.00	32.67 ± 2.33	70.00 ± 0.00	8.51 ± 0.46	61.86 ± 0.95
		Mean ADD		24.11	56.78	126.78	135.29	197.15
27								
	Equine		9.68 ± 0.24	14.17 ± 2.83	25.50 ± 0.00	59.50 ± 4.91	10.63 ± 1.74[4]	61.63 ± 3.25
		Mean ADD		23.85	49.35	108.85	119.48	181.11
	Porcine		9.21 ± 0.41	14.17 ± 2.83	31.17 ± 2.83	68.00 ± 4.91	9.82 ± 0.28	62.21 ± 1.10
		Mean ADD		23.38	54.55	122.55	131.83	194.04

[1]base temperature = 10°C; [2]DD = degree days; [3]ADD = accumulated degree days; [4]n = 2

Table 2. Sex ratio, degree days for prepupal and pupal development and longevity of male and female *Cochliomyia macellaria* (n = 3) on equine and porcine muscle at three temperatures[1].

			Longevity (DD)	
Temperature	Tissue	Sex % Ratio (♂:♀)	♂ ± SE	♀ ± SE
21				
	Equine	44.35:55.65	39.99 ± 1.70	39.95 ± 1.16
	Porcine	52.14:47.86	43.63 ± 0.18	43.27 ± 0.54
24				
	Equine	52.86:47.14	26.24 ± 4.73	31.24 ± 2.87
	Porcine	53.62:46.38	35.73 ± 2.20	36.17 ± 3.70
27				
	Equine	33.93:66.07	22.93 ± 0.45	22.19 ± 0.72
	Porcine	50.45:49.55	26.39 ± 1.33	26.83 ± 0.61

[1]base temperature = 10°C; [2]DD = degree day

VITA

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